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EDITORIAL COMMENT

Tissue Rings PLOD Out a Second Hit in Becker Muscular Dystrophy*

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Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), together called the dystrophinopathies, are a group of X-linked recessive disorders leading to weakness, muscle wasting, and premature death. DMD and BMD are caused by mutations in dystrophin, a central component of the dystrophin-glycoprotein complex linking extracellular, transmembrane, and cytoskeletal elements to stabilize the plasma membrane in areas of high mechanical stress of striated muscle cells. Patients with DMD have nonfunctioning dystrophin, leading to membrane weakening, myocyte necrosis, and early symptoms. Patients with BMD have partially functioning dystrophin and therefore have a milder phenotype.

Dilated cardiomyopathy is a common manifestation in both DMD and BMD, but presentations vary considerably. With advances in respiratory and musculoskeletal care, heart failure has become the most common cause of death in patients with muscular dystrophy. In cardiomyocytes (CMs), weakening of the sarcolemma and disruption of normal t-tubular structure leads to elevated intracellular calcium and myocyte loss. Thinning of the myocardium and ventricular dilation are commonly seen, with cardiac involvement in nearly all patients with DMD by 18 years of age and severe symptoms by the third decade of life. Even female carriers of DMD and BMD often develop cardiac abnormalities detectable by magnetic resonance imaging, with BMD typically manifesting a milder cardiac presentation.[1] In female carriers of BMD, the progression of cardiomyopathy is slower and rarely leads to end-stage heart failure. The mechanistic underpinnings for the range of cardiac presentations from asymptomatic to early need for advanced heart failure therapies is not fully understood and allows deeper investigation into the way mutations in DMD interact with additional patient or environmental factors to contribute to heart failure.

In this issue of *JACC: Basic to Translational Science*, Kameda et al[2] explore the pathogenesis of severe heart failure in a BMD carrier. They identify a second hit mutation in procollagen-lysine, 2-oxoglutarate 5 dioxygenase 3 (PLOD3) and demonstrate functional consequences in patient-derived induced pluripotent stem cell (iPSC) CM tissue rings. The BMD carrier had a heterozygous in-frame deletion of exons 45 to 48 in DMD corresponding to the rod domain near hinge 3 of the dystrophin protein. Her sister, with the same mutation but without cardiomyopathy, served as an opportune control. This case was unusual in that although deletions in this region have been reported as a hot spot for dilated cardiomyopathy, it has not been shown to occur in carriers without skeletal symptoms.[3] After ruling out X chromosome inactivation as a potential cause for her cardiomyopathy, the investigators proceeded to screen the proband and her unaffected sister with a panel of genes related to cardiovascular disease, identifying a heterozygous stop-gain mutation in PLOD3 only in the proband. PLOD3 is the gene encoding lysyl hydroxylase 3 (LH3), an enzyme required for post-translational modification of collagens, and associated with connective tissue disorders. To further investigate the contractile consequences of the PLOD3 mutation in the context of Δ45-48 DMD, 2 isogenic iPSC-CM lines were generated from the patient: one clone containing an inactivated Δ45-48

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DMD allele (WT-DMD iPSC) and the other containing an inactivated wild-type allele (Δ45-48 DMD iPSC), both with haploinsufficiency of PLOD3. A third iPSC line was created by correcting the PLOD3 in the Δ45-48 DMD line. In self-organizing tissue rings (SOTRs), fabricated using iPSC-CMs from the proband, loss of PLOD3 was associated with lower collagen expression and less stiff tissues. The loss of stiffness was compounded by the Δ45-48 DMD mutation and partially rescued by correction of PLOD3. Similarly, the presence of both mutations led to reduced active force generation that was nonsignificantly improved by correction of the PLOD3 mutation.

This study represents an important demonstration of how a potential pathogenic second gene hit may have functional consequences. The 2-hit hypothesis in cardiomyopathy is the notion that a stressor (eg, chemotherapeutic agents, exaggerated preload in pregnancy, alcohol) can reveal or exacerbate underlying genetic risk (eg, titin truncation variants, lamin mutations) for heart failure. Although this has typically been described on an observational level, the investigators here use a gene survey and genetic manipulation in iPSC-CMs to implicate PLOD3. Beyond identification, this methodology also allowed mechanistic dissection of the role of both genetic hits. Future studies on second hits to reveal risk-conferring genetic variants will need to incorporate not only gene profiling but also biochemical and mechanical inputs.

Importantly, Kameda et al[2] used 3-dimensional tissue force measurements to determine the functional impact of Δ45-48 DMD and PLOD3 haploinsufficiency. The use of such a platform serves as a framework to causally link genetic variants to tissue phenotype. A length-tension protocol enabled both the measurement of maximum active force development and stiffness. Given the effects of these mutations on the cytoplasmic dystrophin complex and collagen network, these mutations offer opportunities to delineate extracellular and intracellular contributors of tissue mechanics. SOTRs, which rely on the cells to secrete and crosslink their own extracellular matrix (ECM), rather than using laboratory-purified collagen, will be a fitting tool for more detailed investigation of PLOD3 on collagen network composition and structure.

The use of isogenic control subjects is paramount to obtaining interpretable results. The investigators creatively took advantage of differential X chromosome inactivation and gene editing to generate identical iPSC-CM lines except for PLOD3 and DMD gene status. However, the wild-type iPSC-CM line was received from a separate donor, so interpretations of comparisons with this group are less straightforward. Differences in the amount of Δ45-48 DMD provide interesting opportunities for exploration. For example, with the 2 otherwise equivalent cell lines, simple cell-mixing studies in SOTRs can enable studies defining the dose-dependent relationship between Δ45-48 DMD and force generation.

More generally, this paper foreshadows how ongoing advances in genetics, cell engineering, and tissue engineering will be combined to yield novel and powerful platforms for understanding cardiomyopathies and advancing targeted therapeutics. SOTRs fit within a larger convergence of cardiac tissue-engineering techniques around intermediate-scale rings or strips, compacted against a mechanical load.[4] Engineered tissues are typically straightforward to fabricate and multiplexable, facilitating medium- to high-throughput experimentation. Combination with patient-derived cell lines and genetic manipulation enables individualized genotype-phenotype correlation, including appropriate control subjects, enabling more reliable mechanistic insights. The incorporation and manipulation of ECM components (eg, stiffness) allows the study of the interaction between cardiomyocytes and their surroundings. Importantly, elongated, anisotropic intermediate-scale cardiac tissues, mimicking classical trabecular preparations, are a functional unit convenient for physiological and rigorous mechanical testing. This includes tissue-scale rheological measurements and the derivation of load-dependent and load-independent contractile parameters. Rigorous mechanical testing, including assessments of passive and active force, force velocity, and stress relaxation may reveal further details of ECM sensing by cardiomyocytes. The versatility of more sophisticated platforms, including the capacity to manipulate biomechanical inputs and incorporate potentially toxic or therapeutic agents, will provide diverse opportunities to explore the mechanisms of many “2-hit” scenarios associated with clinical cardiomyopathies.

In summary, the study by Kameda et al[2] shows how a second hit in PLOD3 may contribute to reduced cardiac function and tissue stiffness in Δ45-48 DMD iPSC-CM tissues. Future understanding of the details in the interaction among PLOD3, collagen structure, and mechanotransduction through the dystrophin-glycoprotein complex to the contractile

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apparatus will help deepen understanding of the cardiomyocyte-ECM interaction.

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